Anti-cancer Therapies in High Grade Gliomas

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Abstract: High grade gliomas represent one of the most aggressive and treatment-resistant types of human cancer, with only 1–2 years median survival rate for patients with grade IV glioma. The treatment of glioblastoma is a considerable therapeutic challenge; combination therapy targeting multiple pathways is becoming a fast growing area of research. This review offers an up-to-date perspective of the literature about current molecular therapy targets in high grade glioma, that include angiogenic signals, tyrosine kinase receptors, nodal signaling proteins and cancer stem cells related approaches. Simultaneous identification of proteomic signatures could provide biomarker panels for diagnostic and personalized treatment of different subsets of glioblastoma. Personalized medicine is starting to gain importance in clinical care, already having recorded a series of successes in several types of cancer; nonetheless, in brain tumors it is still at an early stage.

INTRODUCTION

Glial-derived brain tumors include astrocytomas, oligodendrogliomas and ependymomas, among others. Astrocytomas are further classified, according to 2007 World Health Organization into four grades: I and II of low grade gliomas, III and IV malignant high-grade gliomas, with poor prognostic [1]. Grade IV gliomas, or glioblastoma (GBM) represent one of the most aggressive and treatment resistant types of human cancer. With current treatment protocols, patients with GBM have a survival rate of only 1–2 years after diagnosis [2]. Despite the continuous development of new chemotherapeutic agents, brain tumors remain resistant to therapy, particularly chemotherapy, partially because of the high expression of ABC transporter proteins in cancer stem cells [3]. New approaches regarding classical and new therapeutic targets involving angiogenic signals, signaling protein-protein interactions, stem cell targets and crosstalk between all of them are discussed in the following sections.

CONVENTIONAL CURRENT THERAPY OPTIONS

Conventional treatment strategy usually includes standard surgical removal of the tumor, followed by radiotherapy – 40Gy regimen for whole brain radiation therapy and 15Gy regimen for local therapy [4]. Whole brain radiation therapy, paired with surgery, has the best results concerning brain control. Studies show that radiotherapy can be coupled with adjuvant chemotherapy [5]. Temozolomide (known as Temodar in the United States and Temodal in Europe) is an alkylating agent with proven antitumor activity. This agent acts by reducing the levels of O-methylguanine-DNA methyltransferase (MGMT), a DNA repair enzyme. Determination of MGMT methylation status can identify a subgroup of patients responsive to temozolomide treatment. German Neuro Oncology Group (NOA) assessed the potential benefits of using temozolomide in treatment of gliomas. Their trials showed that two-year survival rate was 26.5% with radiotherapy plus temozolomide and 10.4% with radiotherapy alone [2]. Current recommendations state that temozolomide is particularly useful in young patients with MGMT promoter methylation [6]. Overall, the 2 year survival rate for patients treated with temozolomide was increased to 27% while in patients with MGMT promoter methylation the survival rates were close to 49%; the average survival exceeded 70 months [7]. In some cases of favourable prognosis, the survival rates were longer than 5 years post-therapy [8]. Patients older than 70 years with good neurologic performance are also recommended for radiotherapy together with temozolomide. Temozolomide was also better tolerated than other chemotherapeutic agents such as vincristine. It is still unclear if treatment with temozolomide alone is more effective than radiotherapy [8, 9]; nevertheless, their association is the current approach of these aggressive tumors.

ANTIANGIOGENIC THERAPIES

GBM is among the most highly vascularized of all malignancies and relies upon angiogenesis for growth and his-

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tological progression. Therefore, antiangiogenic therapy is currently considered an attractive targeting therapy for malignant gliomas [10-12].

a) VEGF and VEGFRs Directed Monoclonal Antibodies

The vascular endothelial growth factor (VEGF) family and its receptors seem to be the central signaling pathway of glioma angiogenesis. The formation of abnormal tumor vasculature and glioma cell invasion are believed to be the major factors responsible for the resistace of gliomas to treatment [12]. The recently demonstrated high vascularity of brain tumors through the secretion of high levels of VEGF – the key mediator of angiogenesis - [12, 13] led to the use of VEGF antagonists as a therapeutic target in glioma therapy [14, 15]. Tumor VEGF expression is hypoxia-driven via the HIF-1(hypoxia-inducible transcription factors) pathway, but can be activated independently by different tumor cell mutations [16, 17].

The VEGF receptors, VEGFR-1 and VEGFR-2, are both overexpressed in gliomas, being activated by VEGF-A, which is up-regulated in glioblastoma and is produced by multiple cell types, including the tumor, stromal, and inflammatory cells [12]. VEGF-A can stimulate both physiological and pathological angiogenesis, being a ligand for the two receptor tyrosine kinases VEGFR-1(Flt-1) and VEGFR-2(KDR/Flk-1) [17]. The activation of different signaling pathways including Ras/Raf/mitogen-activated protein kinase and phospholipase C-γ/ protein kinaseC induced by VEGFR regulate endothelial cell proliferation and migration. VEGFR-2 is mainly expressed in vascular endothelial cells, where it directly transduces most of the mitotic signals that result in angiogenesis. The actions of VEGF are strongly associated with the irregular and innefficient blood flow, resulting in vasogenic brain edema. Enhanced vascular permeability is induced by VEGF through the MAPK signaling cascade by rearranging cadherin/catenin complexes and perturbing the adherence junctions between endothelial cells. Bevacizumab (Avastin; BV) is the most extensively tested of the antiangiogenic agents and has received approval in the United States as monotherapy for the treatment of recurrent glioblastoma. However, its benefits and antiangiogenic mechanisms of action remain under intense study, since multiple clinical trials with bevacizumab have demonstrated impressive improvements in progression-free survival in both recurrent and newly diagnosed glioblastoma, but these studies have not identified an overall survival benefit compared with historical control [18]. Several mechanisms of action have been suggested for the anti-GBM effect of bevacizumab, including direct inhibition of tumor-associated angiogenesis, a direct anti-GBM effect on VEGF receptor-expressing GBM cells, disruption of the glioma stem cell microvascular niche, and improved vascular function or normalization [19]. However, GBM appears to adapt rapidly to antiVEGF therapy, resulting in rapid tumor growth progression without improvement in overall survival. Most malignant gliomas have coactivation of multiple tyrosine kinases, as well as redundant signaling pathways, thus limiting the activity of single agents [20]. The mechanisms of antiangiogenic escape is probably induced by a compensatory upregulation of the tumor due to the VEGF blockade through alternate growth factors and signaling pathways,

indicating the combination protocols as being of maximal benefit. More, co-option of normal host capillaries by infiltrating tumor cells might be involved in GBM invasiveness and neovascularisation. Bevacizumab showed initial, dramatic promise for advanced malignant glioma in combination with irinotecan (CPT-11) [21]. In addition, unexpected relationships between the dose of antiangiogenic agents and coadministered drugs indicate that high doses of antiangiogenic agents may actually have more negative consequences than lower doses [18]. The potential tumor "escape" is an important issue in GBM treatment when specific, indirect antiangiogenic agents are delivered individually. There is also evidence that anti-VEGF therapy may convert some advanced gliomas to a more invasive, less angiogenesis-dependent phenotype [22]. Although the significance of VEGF expression is still under investigation, it has been hypothesized that VEGF ligands promote tumor growth not only in a paracrine manner, but also in an autocrine manner.

b) Fibroblast growth factor/Fibroblast Growth Factor Receptor

Besides VEGF, angiogenesis in malignant glioma is also induced by basic fibroblast growth factor (bFGF), platelet-derived growth factor (PDGF), angiopoietin-2 (Ang-2), stem cell factor, and hepatocyte growth factor/scatter factor through different signaling pathways [16].

Disruption of Fibroblast Growth Factor Receptor (FGFR) activity or binding to Fibroblast Growth Factors (FGFs) by a tetracycline-regulated suppression system or suramin, respectively, has demonstrated growth delay in orthopic glioma models. Multi-targeted kinase inhibitors with activity against FGFR are in preclinical development [17].

Recently, thalidomide was reported to have an antiangiogenic activity by inhibiting bFGF, which can be exploited as an antitumor drug. Lenalidomide, a potent structural and functional thalidomide analogue, has antiangiogenic, anti-inflammatory, and immunomodulatory activities in preclinical studies [17].

c) Platelet Derived Growth Factor Receptor α

Platelet derived growth factor receptor α (PDGFR α) is a receptor with tyrosine kinase activity (RTK) shown to be overexpressed, amplified, mutated, or truncated in glioblastomas, and is particularly found to be overexpressed in pediatric GBM [23]. A recent study on PDGFR α abnormalities in diffuse gliomas states that PDGFR α point mutations were observed exclusively in grade IV gliomas and high-level PDGFR α amplification was associated with PDGFRA overexpression, high malignancy grade, and older patient age [24].

d) Others

Invasion of ECs (endothelial cells) is an essential event during angiogenesis, as this process involves degradation of the basement membrane and underlying interstitium. The matrix metalloproteinase (MMP) family of enzymes is considered primarily responsible for extracellular matrix degradation. MMP-2 and -9 have been shown to play an important role in neoangiogenesis and tumor vascularization in gliomas. Therefore, the inactivation of MMPs could be an
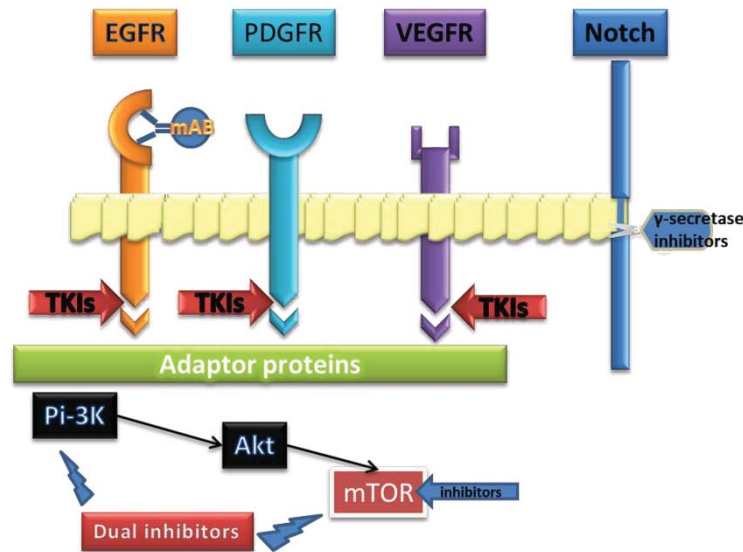
Fig. (1). RTK-PI-3K and Notch molecular therapeutical targets in glioblastoma.
EGFR – epidermal growth factor receptor; PDGFR – platelet-derived growth factor receptor; VEGFR – vascular endothelial growth factor receptor; TKIs – Tyrosine kinase inhibitors

antiangiogenic treatment option. Intraperitoneal administration of an anti-MMP agent, SI-27, limited tumor angiogenesis to a level similar to that found in the normal contralateral hemisphere and successfully prolonged survival in a clinically relevant glioma model. PEX is a 210-amino acid fragment of MMP-2 and it corresponds to the hemopexin domain of MMP-2. PEX binds to integrin αvβ3 and is thought to competitively inhibit the binding of MMP-2 to integrin αvβ3 [17]. Another potentially promising antiangiogenic agent for GBM is cilengitide (EMD121974), a selective inhibitor of the αVβ3 and αVβ5 integrins, cell surface adhesion molecules that facilitate endothelial proliferation and migration through the extracellular matrix, and which are highly expressed in malignant gliomas [21]. There are several studies testing the potential therapeutic efficacy of endogenous inhibitors, such as angiostatin, endostatin, PEX, pigment epithelial-derived factor (PEDF) and thrombospondin (TSP)-1 and -2, in animal models of malignant glioma [17].

TARGETING EGFR AND PI-3K/AKT/MTOR PATHWAY

Tyrosine kinases receptors are transmembrane proteins involved in cell proliferation and differentiation, cell survival and metabolism, cell migration, and cell-cycle control [25]. Mitogenic signals received from cytokines and growth factors are further sent inside the cell by receptor dimerization and cross-activation of C-terminal kinase domains.

a) Epidermal Growth Factor Receptors (EGFRs)

For EGFRs activation is "receptor-mediated" and activating ligands contact two distinct sites within a single receptor molecule, rather that cross-link two receptors, like other classes of receptors [26]. Activation of these receptor tyrosine kinases leads to the formation of secondary adaptor complexes, recruitment of PI-3K to the plasma membrane through the binding of the SH2 domain of p85 to the phospho-tyrosine residues on adaptor proteins, and the release of

the p110 catalytic subunit from inhibition by the regulatory subunit (Fig. **1**). The major signaling arm of PI-3K is Akt serin/threonin kinase also known as protein kinase B or PKB. There are three highly related isoforms of Akt (Akt1, Akt2, and Akt3) that regulate cell growth through its effects on the TSC1/TSC2 complex and mTOR pathways, as well as cell cycle and cell proliferation through direct action on the CDK inhibitors p21 and p27, and indirect effect on the levels of cyclin D1 and p53 [27]. Akt is a major mediator of cell survival through direct inhibition of pro-apoptotic signals such as the pro-apoptotic regulator Bad and the Foxo and Myc family of transcription factors [28].

Overexpressed or mutated RTKs have been documented in many tumors: lung cancer [29], squamous cell carcinoma [30], breast cancer [31], ovarian cancer [32]. One of GBM pathogenic hallmark is RTKs amplifications and a recent study showed that in 50% of cases amplification of at least one of the 51 RTKs investigated was present. The most commonly amplified RTK gene was EGFR (41% of the cases) followed by PDGFRA (10%) in a mutually exclusive fashion [33]. So far, there are several RTKs inhibitors used in clinical practice for treatment of various neoplasms [34].

Numerous studies have identified epidermal growth factor receptor as a common genetically altered gene in primary GBM [35-37], by: *i)* amplifications [35, 38] *ii)* exon deletions affecting either the extracellular, either cytoplasmatic domain [39-41] *iii)* point mutations within the extracellular domain of EGFR [42]. These genetic alterations have been shown to lead to oncogenic activation of the mutant receptor independent of ligand stimulation and, consequently, induce cellular transformation. In addition, various exon deletion mutations including exon 25–27 and exon 25–28 deletion mutations, which result in the truncation of the C-terminal domain of EGFR, have been identified in GBM patients although their oncogenic potential has not yet been characterized. Furthermore, EGFR gene amplification and/or EGFR protein overexpression commonly occur in approximately 50% of GBM patients, suggesting that an increased abun-

dance of the EGFR may also be responsible for tumorigenesis in primary GBM [43]. There are two categories of molecular therapies directed towards RTKs: monoclonal antibodies and tyrosine kinase inhibitors (TKIs). The most studied antibody is cetuximab, which functions to prevent EGFR-mediated signal transduction by interfering with ligand binding and EGFR extracellular dimerization. Two phase II clinical trials report either no efficacy improvement in combined therapies including cetuximab [44], either an increase in overall survival, but only in wild-type EGFR amplified GBM [45].

Tyrosine kinase inhibitors (TKI) are small molecules that act as reversible or irreversible adenosine triphosphate (ATP) analogues. They inhibit EGFR signaling by competing and binding with ATP binding pockets on the intracellular catalytic kinase domain of RTKs, thereby preventing autophosphorylation and activation of several downstream signaling pathways [46]. The TKIs used in tumor research and clinical trials are listed in Table **1**. However, in spite of various RTKs being mutated or altered in the majority of GBMs, clinical studies have not been able to demonstrate efficacy of molecular targeted therapies using tyrosine kinase inhibitors in GBMs. Activation of multiple downstream signaling pathways has been implicated as a possible mean by which inhibition of a single RTK has been ineffective in GBM [34].

Regarding TKIs, Chakravarti et al. reported no overall survival benefit for patients treated with gefitinib + RT when compared with a historical cohort of patients treated with RT alone [47] and a recent phase II clinical trial for GBM therapy, erlotinib was well tolerated, but only demonstrated a modest effect over placebo [48].

b) PI-3K/Akt Inhibitors

Several pan-PI-3K inhibitors have been developed, *e.g.,* wortmannin, Ly294002, staurosporine, quercetin, demethoxyviridin and PI-103. Wortmannin and demethoxyviridin are potent, irreversible, but non-selective inhibitors of all PI-3K. LY294002 and quercetin are reversible and potent PI-3K inhibitors, but non-selective and also bind other members of the PI-3K family [49]. LY294002 has proven to be a very useful research tool to delineate PI-3K signaling in the cells, but its poor pharmacological properties, such as limited stability, have precluded clinical development of this molecule [50]. In 2008 a water soluble, with favourable pharmacokinetics, and well tolerated LY294002 derivate - SF1126- was reported as a viable pan PI-3K inhibitor for phase I clinical trials in cancer [51]. Up-to-date use of SF1126 in several phase I trials have been reported to have encouraging results [52, 53].

A new series of PI-3K inhibitors, which selectively target different PI-3K isoforms, that may help minimize the toxic side effects of general pathway inhibition, were synthesized [49]. The PI-3K isoform most successfully specifically targeted to date is the p110δ subunit [54].

Akt is a downstream effector of the PI-3K pathway, which is commonly up-regulated in the majority of GBM tumor samples and cell lines, and help glioma cells grow uncontrolled, evade apoptosis, and enhance tumor invasion. Akt represents a nodal point in this pathway, which allows

for amplification of growth signals, thereby making inhibition of Akt an attractive target for GBM therapy [55]. Akt also regulates protein synthesis and cell growth through activation of mTORC1 and subsequent phosphorylation of ribosomal p70S6 kinase (S6K1) and eukaryotic initiation factor 4E binding protein 1 (4EBP1) [54]. Akt has also been implicated as an important target of phosphatase and tensin homologue (PTEN) and thus, the dysregulation of Akt seems to be an important consequence of the loss of PTEN function [56].

PTEN is a phospholipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate and inhibits PI-3K-dependent activation of Akt. The mutation or loss of PTEN leads to constitutively activated Akt [57].

c) mTOR

mTOR links growth factor signaling through PI-3K to energy and nutrient status, protein translation, autophagy, and tumor cell metabolism, thus acting as a critical integrator that regulates tumor growth, survival and, potentially, cancer drug resistance [58]. mTOR is the downstream effector of PI-3K/Akt pathway and, as a consequence, an attractive therapeutic target for GBM. Of the two functionally different complexes (mTORC 1 and 2), rapamycin addresses only mTORC1 and failed as a treatment option [59]. Recent research results have highlighted the therapeutic potential role for ATP-competitive inhibitors of mTORC1/mTORC2, mTOR kinase inhibitors Torin, Ku-0063794 and pp242, which block both mTOR signaling complexes [60].

Targeting of mTORCs in glioblastoma is further justified by research results showing that secreted factors from brain endothelial cells maintain glioblastoma stem-like cell expansion through the mTOR pathway [61].

Preclinical evaluation of dual PI-3K/mTOR inhibitors, such as PI-103 and NVP-BEZ235 have demonstrated efficacy for these agents in blocking the growth of glioblastoma (GBM) cells in vitro and in vivo, therefore NVP-BEZ235 and other dual inhibitors are being evaluated in early clinical trials [60]. A dual PI-3K/mTor inhibitor, NVP-BEZ235, elicited a prodifferentiation effect on A172 CSLCs. Moreover, A172 CSLCs, which were induced to undergo differentiation by pretreatment with NVP-BEZ235, exhibited a significant decrease in their tumorigenicity when transplanted either subcutaneously or intracranially. Importantly, similar results were obtained when patient-derived glioblastoma CSLCs were used [62].

By targeting the Notch pathway, it may be possible to interfere with these processes leading to a better treatment outcome for patients, especially those with high-grade astrocytic gliomas. In addition, if Notch signaling plays a role in bCSC, and if these cells are crucial for glioma maintenance, it may be possible to target these tumor-initiating cells by inhibiting the Notch pathway. Presently, γ secretase inhibitor RO4929097 is included in clinical trials, as single agent therapy in combintion with conventional surgery (ClinicalTrials.gov Identifier: NCT01269411), or associated with temozolomid and radiotherapy (ClinicalTrials.gov Identifier: NCT01119599) or with bevacizumab (ClinicalTrials.gov Identifier: NCT01189240), on several types of gliomas.

Table 1. Mode of action of signaling pathway-targeted inhibitory compounds.

Signaling Pathway	Compound Name	Mode of Action
VEGF - directed monoclonal antibodies	Bevacizumab	specifically binds to VEGF-A and prevents ligand-receptor interaction [63]
	Aflibercept	binds to all isoforms of VEGF-A with high affinity [64]
VEGFR – directed monoclonal antibodies	Icrucumab	human IgG1 monoclonal antibody directed against human vascular endothelial growth factor receptor 1[65]
	Ramucirumab	humanized monoclonal anti VEGFR-2 [64]
VEGFR small molecules kinase inhibitors	Sorafenib	inhibits the proangiogenic VEGFR-1, VEGFR-2, VEGFR-3, and PDGFR-β tyrosine kinases in biochemical assays in vitro [66]
	Sunitinib	VEGF receptors-1, -2, and -3, PDGFR-α and -β, c-KIT, the receptor tyrosine kinase receptor encoded by the ret proto-oncogene (RET), and fms-like tyrosine kinase 3 (Flt3) [67]
PDGFR – small molecules kinase inhibitors	Imatinib mesylate	inibitor of PDGFR, ABL, and c-KIT [68]
	Tandutinib	inhibitor of PDGFR, FLT3, and c-KIT tyrosine kinase activity [68]
EGFR - directed monoclonal antibodies	Cetuximab	binds to the second (L2) domain of EGFR thereby blocking its downstream signaling by prompting receptor internalization and encumbering ligand-receptor interaction [46]
	Panitumumab	blocks the binding of both EGF and TGF-alpha to various EGFR [69]
EGFR small molecule kinase inhibitors	Gefitinib	orally active low-molecular-weight EGFR inhibitor with selective tyrosine kinase activity but not serine-threonine kinase inhibitory activity [70]
	Erlotinib	anti-proliferative effects, cell-cycle arrest and apoptosis [71]
	Lapatinib	reversible and specific RTK inhibitor of both EGFR and HER2 as well as against AkT [72]
	Canertinib	orally active low-molecular-weight irreversible pan-EGFR family TKI [46]
PI-3K inhibitors [73]	SF1126	Pan-class I PI-3K, mTOR, DNA-PK
	PX-866	Pan-class I PI-3K
	GDC-0941	Pan-class I PI-3K
	NVP-BEZ235	Pan-class I PI-3K, mTOR
	XL147	Pan-class I PI-3K
	XL765	Pan-class I PI-3K, mTOR
	D-87503	Pan-class I PI-3K, ERK2
	GSK615	Pan class I PI-3K
	CAL101	PI-3Kδ
Akt inhibitors	GSK690693	inhibits all three isoforms of Akt and some related AGC family kinases [74]
	Akt VIII	electivity towards Akt1 and Akt2 [75]
	MK-2206	targets all three Akt isoforms [54]

Table 1. contd….

Signaling Pathway	Compound Name	Mode of Action
mTOR inhibitors [60]	Rapamycin	mTORC1 inhibitor
	Rapamycin analogs (rapalogs temsirolimus and everolimus)	mTORC1 inhibitors
	Torin	mTORC1,2 inhibitor
	Ku-0063794	mTORC1,2 inhibitor
	pp242	mTORC1,2 inhibitor
dual PI-3K/mTOR	PI-103	inhibits both PI-3K and mTOR kinase activity (in mTORC1 and mTORC2) [76]
	GDC-0941	more selective towards PI-3K then mTOR and is currently in Phase I clinical trials [77]
gamma-secretase/Notch signaling	RO4929097	Targets p75NTR to inhibit brain tumor initiating cells and recurrent invasive gliomas [ClinicalTrials.gov Identifier: NCT01269411] [78]

MIRNAs As ANTI-ONCOGENIC THERAPY IN GBM

One of the most promising therapeutic targets in cancers are microRNAs (miRNAs). miRNAs are also associated with various types of human cancers - and are sometimes referred to as "oncomirs." [79]. Some miRNAs are thought to have oncogenic activity, while others have tumor suppressor activity. Oncogenic miRNAs are up-regulated in cancer and contribute to its pathology through various mechanisms such as targeting tumor suppressor genes. Other miRNAs are considered to have tumor suppressor activity and are down-regulated in cancer [80]. However, these distinctions may not be so strict, suggesting that some miRNAs may express either activity, depending on the biological context and tissue type [81]. Recently published studies unveil the potential role of miRNAs in cancer stem cells, suggesting their role in resistance to treatment [82].

Several miRNAs have been identified in glioblastoma (GBM), as playing a key role in carcinogenesis and tumor progression [83, 84] by regulating proteins with oncogenic and tumor suppressive properties [85]. Four miRNAs were constantly found dysregulated in gliobastomas: miR-21, miR-196, miR-10b, miR-128-1, and miR128-2 and are potential participants in glioma genesis [82].

Five clusters of GBM based on miRNA expression profiles were identified by Kim *et al*. (2011); based on these expression profiles they seemed to predict clinical outcomes more accurate than mRNA profiles [86]. However, a stronger association miRNA-mRNA expression signatures was identified [87, 88] and a better understanding of interaction between miRNA and mRNA would lead to improvements in stratification of GBM [82].

MiR-21 was the first oncomiR identified in GBM [89, 90] and represents a unique miRNA overexpressed in almost all types of tumor investigated to date [82]. Its overexpression is driven by the transcription factors STAT3 and AP-1 [91, 92], and its relevant targets appear to include tumor-suppressive genes such as PDCD4, PTEN, TPM1, RECK, and TIMP3 [93-96].

A recent *in vivo* study revealed that miR-21plays a crucial role in various steps of tumor progression [97]. MiR-21 acts as an antiapoptotic factor that targets a network of p53, transforming growth factor (TGF)-β, and mitochondrial apoptosis tumor suppressor genes in glioblastoma cells [90, 98].

High expression of **miR-196** it was shown to be associated with shorter overall survival among glioblastoma patients [99]. When compared to other overexpressed miRNAs in glioblastoma, miR-196a and miR-196b appear extremely high expressed, therefore are considered to be involved in malignant transformation of gliomas.

Several reports showed that **miR-10b** is commonly upregulated in glioblastoma tissues, glioblastoma cell and stem cell lines as compared to normal human tissues or astrocytes [86, 100-102] and its presence was associated with the invasion and migration of glioma cells [103]. Other studies assigned to miR-10b important roles in regulating cell cycle and programmed cell death, by regulating Bim, TFAP2C, p16, and p21. Overexpression is detected in most of glioblastoma tumors but is absent in normal brain and is significantly associated with tumor invasion factors, uPAR, and RhoC [99]. It was associated with patient survival [101].

MiR-128, a molecule found in normal brain associated with differentiated neurons [89, 104], is downregulated in glioblastoma compared to adjacent brain; this result in a decrease in self-renewal glioma stem cells through Bmi-1 downregulation [100]. miR-128 expression is lower in high-grade gliomas than in low grade gliomas, reduced levels of this molecule being associated with dedifferentiation and aggressiveness of malignant gliomas via EGFR/PDGF/AKT signaling [84]. It is notable that *in vivo* miR-128 was capable of growth suppression in glioma initiating cell, thus supporting its potential role in therapy [105].

MiRNAs–biomarkers in GMB

A growing number of studies show the potential use of plasma or serum miRNAs (extracellular miRNAs) as biomarkers for detection, identification, classification, prognostic predictors and response to therapy in cancers [106]. Expression of these molecules specific in different tissues, different expression levels in tumor versus normal tissue [107-109], stability of miRNAs in plasma or serum [110] are arguments which sustain their appealing proposal.

MiRNA in GBM Therapy

Besides their roles as biological markers for diagnosis, cancer prognosis, and therapeutic response, miRNAs are promising anticancer therapeutic agents [85]. The major constraint that limits the use of systemic delivery of miRNA in glioblastoma treatment is low permeability of this barrier; local intratumoral administration should be considered instead [85].

An encouraging local delivery strategy capable of improving recent tratments is convection-enhanced delivery (CED) – a technique which involves continuous low pressure infusion via catheter, "creating a pressure wave that drives the infusate to replace brain interstitial fluid over what can become a large field around the catheter tip" [111].

Reports show that a nanoparticle based delivery system might provide an improved strategy for miRNA therapy in brain cancer treatment. Some authors suggest the use of viral vector-based therapy to overexpress tumor-suppressive miRNAs might be a successful option [112]. Since tumor secreted exosomes contain a large amount of miRNA [113], another miRNA therapy alternative might come from exosome biology [82].

Up to date scientific literature suggests two possible treatment options for glioblastomas with regard to miRNA: substitution of miRNA with tumor suppression function (mimics) and inhibition of miRNAs with oncogenic properties [82]. Replacement therapy has clear advantages over gene therapy due to the difference in miRNA size over conventional genes. Recent studies have reported miRNA replacement for miR-7, miR-34 or miR-128 [100, 114, 115]. miR-34, a key regulator of cancer progression in GMB, is suggested as a possible target for future therapies.

Positive results were noted in preclinical animal studies when GBM miRNA expression and activity were modulated. In a glioma mouse model important decrease in growth tumor was observed after inhibiting miR-10b by intratumoral antimiR injection [101]. Other animal studies also reported tumor reduction following inhibition of oncogenic miR-21 in GBM [116]. MiR-21 and miR-195 down regulation in GBM culture cells sensitize cells to treatment with 5-fluorouracil and temozolomide [117, 118].

Regardless the proposed approach, designing proper delivery systems (for either mimics or inhibitor miRNAs) that will reach the target tissues is still a prerequisite currently under intense investigation. Overcoming the difficulty of delivering therapeutic agents in GBM represents a major challenge to treatment since blood-brain barrier (BBB) is impermeable for many potentially effective molecules; cer-

tain small nonpolar circulating molecules can access the brain by passive diffusion through the BBB or by active transport, but most drugs and chemicals cannot readily cross into the brain.

THERAPY INVOLVING CANCER STEM CELLS IN BRAIN TUMORS

During last years, a great deal of research was directed towards cancer stem cells (CSC) and their role in glioblastoma survival and relapse. Apparently, the heterogeneity of brain tumors reside in the heterogeneity of their CSCs. CSCs are involved in complex mechanisms driven by their microenvironment**,** where the niche is one of the key players. Addressing CSC as the most recent GBM therapy research field, Binello and Germano classified the main directions into direct and indirect targeting [119]. Direct targeting refers to overcoming resistance to standard treatment, blocking CSC functions through EGFR/PI-3K/Akt inhibition and inducing differentiation. Indirect targeting addresses perivascular niche, hypoxic niche and immune evasion.

Increasing Sensitivity to Treatment

CSC have molecular peculiarities, such as expressing multidrug-resistance genes (like ABCG2 and BCRP1, whose function is to aid in the efflux of drugs and in the selective promotion of CSC survival. The expression of multidrug-resistance genes was enhanced in GBM-tumor sphere cells [120]. The identification and isolation of glioblastoma CSCs is based on the expression of stem cell-associated protein CD133 [121, 122]. CD133+ cells isolated from glioblastoma surgical specimens are more resistant to radiation than CD133- cells isolated from the same tumor [123].

It has recently been described that CD133+ glioblastoma CSCs are more radiosensitive in vitro than established glioblastoma cell lines, with a reduced capacity to repair DNA double-strand breaks (DSB), and although they have an intact G2 checkpoint, they lack the intra-S-phase checkpoint [124].

CD133+ CSCs can escape the lethal damage by preferential activation of DNA repair checkpoints, including phosphorylation of the checkpoint proteins Chk1 and Chk2 [123]. Endothelial cells might also promote CD133+ CSCs radioresistance [125]. Interestingly, some studies suggested that aberrant activation of developmental pathways in CSCs, including Bone Morphogenetic Protein (BMP) [126, 127], Notch [128-131], Sonic hedgehog (Shh) [132, 133], PI-3K-Akt-PTEN [134, 135], and EGFR [13, 125, 136], may contribute to CSCs resistance to chemo- and radio-therapy. Due to the complicated mechanisms involved in CSCs resistance to standard chemotherapy and radiotherapy, it is rather difficult to eradicate CSCs by sole therapeutic strategy and thus combined therapeutic strategies are recommended.

It was found that Notch signaling levels were higher in the CD133+ cell fraction in medulloblastoma cell lines [129]. Following Notch blockade with gamma-secretase, CD133+ CSCs decreased, with inhibited proliferation, increased differentiation, and reduced tumorigenicity. CSCs thus seem to be selectively vulnerable to agents inhibiting the Notch pathway. Hedgehog-Gli signaling inhibitor regu-

lates the proliferation of CSCs and can be used in a combinatorial approach to treat the tumor including CSCs, while sparing normal quiescent stem cells in their niches [133]. It has also been found that after treating with Hedgehog-Gli signaling inhibitor, the GBM-derived neurosphere cells lost their tumorigenicity, and the expression of stem cell markers including CD133 were also reduced [132]. More importantly, Hedgehog blockade may target GBM cells which are unaffected by radiation. These results revealed that Hedgehog blockade offers new therapeutic possibilities which can combine with chemo- or radio-therapies.

Akt inhibitors not only sensitize brain CSCs to radiation-induced apoptosis, but can also directly target CSCs.

Because brain CSCs are indeed maintained within vascular niches that mimic the neural stem cell niche [125], these studies highlight the possibility that selective eradication of CSCs may be achieved by a combination of antiangiogenic drugs, which can deplete the tumor blood supply and disrupt stem cell preservation with other chemo- and radio-therapies.

Low level of major histocompability complex I or natural killer (NK) cell activating ligands were detected in CD133+ and CD133- glioma cells, and interferon gamma may partially restore their immunogenicity and potentiate their lysis by NK cells [137]. These results suggest that it is a promising therapeutic strategy to enhance the expression of NK activating ligands in brain tumor cells, especially in CSCs.

Future perspectives on stem cell therapy involving transplant of stem cells as delivery cargos and nanotechnology, will allow personalization of tumor treatment. Furthermore, adaptive therapy, in which the drug dosing or timing varies depending not only on preclinical tumor growth dynamics, but also on clinical tolerance and response are only a few of possible directions to follow in glioblastoma treatment [138].

Blockage of EGFR/PI-3K/Akt Signaling Axe

For the major signaling players involved in CSC such as Notch, BMP, Shh, and others, therapy approaches were sought [127, 129, 132, 133]. The treatment of GBMs and medulloblastoma cells with a Shh pathway inhibitor depletes stem cell-like cancer cells [132]. Cyclopamine inhibits the growth of human glioma and the self-renewal of GBM-bCSCs [133]. Similarly, a Notch pathway inhibitor can decrease the number of stem cell-like cells in medulloblastoma cell lines [129]. In addition, pharmacological Notch blockade using gamma-secretase inhibitors reduces the percentage of cells expressing the stem/progenitor cell markers CD133 and Nestin in glioblastoma neurospheres. Moreover, following Notch blockade, surviving cells are no longer able to efficiently form in vitro colonies or engraft *in vivo* [139]. Fan X also discovered that the reduced proliferation and induction of apoptotic markers in GBM following Notch blockade is associated with phosphorylation changes to Akt and STAT3, suggesting additional pathways that might be synergistically targeted and that cross-talk exists between these pathways. Recently, a monoclonal antibody, OMP-21M18, directed against the N-terminal epitope of Notch ligand delta-like 4 (DLL4) preventing its interaction with Notch-1 and Notch-4 receptors, was included in clinical tri-

als [140]. Perspectives are that pharmacological Notch blockade, either alone or in conjunction with other therapies, will be effective in improving the survival of patients with GBM and other malignant tumors [139].

Differentiation Therapy

The drug that was clinically tested in this respect is all-trans-retinoic acid (ATRA) [141], a drug previously used in promyelocyte leukemia. The mechanism of action is still under study. Several groups have obtained positive results in experimental models. *In vitro*, early-stage mouse embryonic stem cells could be differentiated into neural cells by ATRA [142]. In C6 rat glioma cells, ATRA induced the differentiation toward oligodendrocytic cells [143], while accelerating the differentiation of NSCs into neuron-like cells in neonatal rat striatal neural stem cells [144].

In human cells, ATRA could increase the percentage of neurons in the course of inducing the human embryonic neural stem cells to differentiate by down-regulation of Notch1 expression [145]. All these studies showed the clear possibility of using ATRA to induce differentiation of brain cancer stem cells and to be one of the therapeutical approaches.

Another novel treatment forcing the bCSCs to differentiate into non-dividing cells used BMP4, which lead to the differentiation of NSCs into astrocytes. Transient *in vitro* exposure of bCSCs to BMP4 significantly reduced their number, and the *in vivo* delivery of BMP4 blocked the growth of human GBM cells in the mouse brain [127].

Targeting the Tumor Microenvironment

It has been reported that endothelial cells release soluble factors that stimulate self-renewal and inhibit differentiation and that antiangiogenic therapies inhibit the supply of blood to bCSCs and reduce their number [146]. In xenografted glioma tumors, a combined antiangiogenic and cell cytotoxic drugs reduced the tumor stem cell-like cell fraction [136].

In a recent animal model study, for inducing IFN-alpha and CXCL10/IP-10 in the CNS tumor microenvironment, another approach was published. Administration of polyinosinic-polycytidylic acid stabilized by lysine and carboxymethylcellulose (poly-ICLC), a ligand for toll-like receptor 3 and melanoma differentiation-associated gene 5 (MDA5) in combination with vaccinations targeting CTL epitopes derived from glioma-associated antigens (GAAs) was used. The combination of subcutaneous vaccination and i.m. poly-ICLC administration improved the survival of tumor-bearing mice in the absence of detectable autoimmunity. Authors have started a phase I/II vaccination study using type 1 polarizing DCs loaded with GAA peptides in combination with poly-ICLC in patients with recurrent malignant glioma [147].

In contrast to normal neural stem cells, CSC express high levels of MHC antigens, therefore, active immunotherapy in which a vaccination strategy is used to induce a T cell response specifically against cancer stem cells may be an effective means of exploiting this therapeutic window. The mechanisms and molecules that trigger chemo- and radio-resistance of CSC can be potential targets.

Immunotherapy – Expectation and Prospection

In all types of vaccination, that are recently in the phase of clinical trials in cancers, the challenge is to break tolerance, so that the patient's immune system will recognize cancer cells. Identifying the specific tumor antigens is the first step in an efficient vaccination, followed by the proper immune strategies that help an anti-tumoral response and break all the inhibitory immune mechanisms. Dendritic cells (antigen-presenting cells that stimulate the naïve immune system vaccination) are involved in maintaining self-tolerance [148]. In vaccination studies, DCs have been loaded in culture with specific tumor-associated peptides, tumor RNA and cDNA, tumor cell lysate, or apoptotic tumor cells [149]. When entering the clinical phases, DC vaccination needs to standardize the protocol of DC generation, DC subtype, dose and timing interval of vaccination, route of administration, approaches of antigen loading, and especially, DC maturation [150].

Therefore, in a phase I study using DC vaccine in patients with newly diagnosed high grade glioma was described [151]. DCs cultured from patients' peripheral blood pulsed *ex vivo* with autologous tumor cell-surface peptide were given intradermally and radiological evidence of disease progression after receiving the third vaccination was obtained. When using tumor lysate as an antigen source for vaccinating patients diagnosed with malignant glioma**,** including anaplastic astrocytoma and glioblastoma, interesting results were obtained [151]. In another phase I study with DC vaccination in patients with glioblastoma it has been found that patients who developed systemic antitumor cytotoxicity had longer survival time and all of the patients who had stable/minimal residual disease at baseline generated a positive cytotoxic T-lymphocytes (CTL response) [152].

In mouse experimental model using mouse glioma GL261 neurospheres (GL261-NS), it was proven that CTLs generated by vaccinations with DC pulsed with tumor lysate derived from CSC could efficiently deplete the tumor of its specific CSCs [153]. Showing that GL261-NS has expressed molecules highly relevant for immune recognition, it was postulated that GBM cells from relatively small amounts of tumor tissue could be amplified in vitro as neurospheres and used for treating the relapse [140].

In half of the patients there was one or more tumor-associated antigen (TAA)-specific cytotoxic T-lymphocyte (CTL) clones against melanoma antigen-encoding gene-1, gp100 and human epidermal growth factor receptor (HER)-2. This last finding is not to be intriguing, since the neural crest origin of melanocytes is known. After discovering that SOX2 is an important gene for self-renewal in both normal neural stem cells and brain cancer stem cells, specific CTLs were raised against the HLA-A0201-restricted SOX2-derived peptide (TLMKKDKYTL). These CTLs were capable of lysing glioma cells [154]. Therefore, the glioma-restricted overexpression of SOX2 can indicate this molecule as a target for T cell-based immunotherapy of brain cancer stem cells. CTL vaccination showed promising results in other types of cancer as well [155].

CROSS TALK BETWEEN KEY STEM SIGNALING - ANGIOGENIC AND RTK SIGNALING

In light of recent molecular treatment-acquired resistance or inefficacy, it has been proposed that tumor cell susceptibility to targeted therapeutics is greatly affected by context-dependent oncogene addiction, receptor mutations. In cancer cells networking activation prevails and identification of essential nodal components should be the targeted for sensitivity to cancer therapy [156]. For example, expression of the constitutively active mutant *EGFR*vIII sensitizes tumors to EGFR inhibitors, but only if the PTEN tumor suppressor protein is intact. Amplification of EGFR coupled with low levels of activated Akt were proven more likely to respond to small molecule tyrosine kinase inhibitors [157]. mTORC1 appears to be an effector of EGFR inhibitor resistance through PTEN loss or RTK activation [158] and, as previously discussed, dual EGFR/mTOR inhibition was effective at targeting EGFR-activated PTEN deficient tumors [159]. Now is known that more than one signaling pathway can be activated through receptor signaling and other receptors can be co-activated to maintain downstream pathway activation, despite EGFR inhibition [160]. Furthermore, it was shown that Akt inhibition induced the expression and phosphorylation of multiple RTKs, partly through mTORC1 inhibition and partly through a FOXO-dependent activation of receptor expression [161].

When considering the cross-talk between Notch signaling and the RAS/MEK/ERK and PI-3K/Akt pathways downstream of EGFR and their roles in experimental gliomas, it is tempting to speculate that simultaneous inhibition of several of these pathways could lead to improved treatment of glioma patients. As Notch signaling, and especially Dll-4, is involved in tumor angiogenesis, it is likely that this pathway is involved in anti-VEGF resistance. Indeed, although initially responsive to bevacizumab, Dll-4-expressing U87MG glioma cells continued to grow at the same rate as control-treated tumors after terminating treatment [162]. Blocking Notch signaling by using a soluble form of Dll-4 reduced tumor burden and prolonged survival of the Dll-4 expressing tumors. Most importantly, soluble Dll-4 inhibited growth of both bevacizumab-sensitive and -insensitive tumors indicating that targeting Notch in addition to VEGF would result in improved treatment outcome.

Antagonizing epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) aim more to alter the relationship with the cancer stem cell niche [125, 163].

MicroRNA-451, MicroRNA-486, and MicroRNA-425, as well as microRNA-124 and microRNA-137, have been demonstrated to affect CD133+ CSCs. Furthermore, a combination of MicroRNA-451 and Imatinib mesylate showed a synergistic effect on inhibiting neurosphere formation [164].

CHALLENGES IN BRAIN TUMOR PERSONALIZED MEDICINE

As in so many fields of cancer treatment, glioblastoma therapy also acknwoledge the role of personalized medicine for tumor phenotype-based treatment. The ability to classify tumors into various phenotypic groups based on molecular expression of biomarkers and according stratification of pa-

tient groups are the future of personalized medicine. So far, several molecular features have beeing idenified as candidate biomarkers for GBM, but probably a more suitable approach for most efficacious medication would be the use of an individual proteomic profile. This approach will fortunately lead to significantly improved outcomes for a subset of patients, inspite the dismail results of current standard therapies.

A future perspective for personalized therapy is targeting protein-protein interaction involved in signaling networks specifically altered in different subset of glioma patients.

CONCLUDING REMARKS

Despite the important progress in the treatment of malignant gliomas and in our understanding of the molecular pathogenesis of these tumors, the most appropriate therapy selection is a real challenge and may open real opportunities on the basis of the patient's tumor genotype. Given the current data of clinical trials using molecular targets, such as monoclonal antibodies or small molecule inhibitors, more knowledge is needed in the field of glioblastoma research. Such knowledge might be achieved through proteomic/biomarkers approaches, which would simultaneously identify potential candidates for target therapy. Cross talk between different signaling pathways may partially explain molecular treatment acquired resistance or its inefficacy. New promising investigation areas and therapy targets are the microRNA field and cancer stem cell research. Cancer stem cell cooperation with their niches is determinant in maintaining and expressing stem cell function, along with the activation of many signaling pathways, such as RTKs-Akt, Notch, BMPs, Shh, Hedgehog, Wnt-β-catenin, STAT3, Bmi-1, that can be targeted by future therapies. Therapy addressing CSC in brain tumors comprises differentiation, sensitization of CSC to radio/chemo-therapy, tumor microenvironment targeting, immune therapy. The development of new protocols by combining chemotherapy with immunotherapy to achieve therapeutic synergy will be applicable in brain tumors. We may be entering a new phase in cancer research based on the cancer stem cell paradigm in the following 5 years.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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